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Article

Phytochemical Analysis, Antioxidant and Anticancer Effects of *Clitoria ternatae* Extract on Breast T47D Cancer Cells

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Abstract: Background: Breast cancer is one of the most common and deadly forms of cancer in the world. Cancer is a multi-factorial disease. Genetic factors, environment and lifestyle have a role in the development of cancer. One of the mechanisms of cancer development is when an imbalance between free radicals and antioxidants in the human body occurs. Uncontrolled and excessive amount of free radicals and cause cell damage and uncontrolled cell growth. *Clitoria ternate* is a plant that is often found in Asia and many of the benefits of this flower have been studied. This study aims to determine the phytochemical constituents, antioxidant activity, and cytotoxic activity of Clitoria ternatea against T47D breast cancer cells.

Method: *Clitoria ternatea* in the form of dry powder is macerated in a multi-level manner with n-hexane, ethyl acetate, and ethanol as solvents, producing a *Clitoria ternatea* extract of the respective solvents. Each extract is then evaluated for its phytochemical constituents, antioxidant activity, and cytotoxic activity using phytochemical test, thin layer chromatography (TLC), DPPH assay, and MTT assay respectively.

Results: Phytochemical analysis of *Clitoria ternatea* shows the presence of glycosides, flavonoids, tannins and triterpenoids with TLC revealing the presence of ten phytochemical constituents. DPPH assay reveals that *Clitoria ternatea* exhibits a very active antioxidant activity. MTT assay revels *Clitoria ternatea* has a high cytotoxic activity towards T47D breast cancer cell line with IC50 value ranging from 1.27 μg/mL to 32.38 μg/mL.

Conclusion: Chemical constituents of *Clitoria ternatea* is responsible for the antioxidant and cytotoxic activity towards T47D breast cancer cell line.

Keywords: *Clitoria ternatea*¸antioxidant, phytochemichal, breast cancer, T47D cells, cytotoxicity

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1. Introduction

Cancer is a disease in which cells of the human body undergo abnormal cell growth which can hinder normal organ functions. Cancer cells has the potential to metastasize (spread) to other organs in the body through blood or lymphatic vessels, causing more damage to the patient. According to the World Health Organization (WHO), there are 18 million new cases of cancer in 2018 and a total of 9.5 million casualties due to cancer in 2018.[1] This data revealed that breast cancer is one of the most common form of cancer and also one of the deadliest. In 2018, there are a total of 2,088,849 new cases of breast cancer globally and 626,679 death caused by breast cancer. Breast cancer is a global issue that has

also affected Indonesia. According to GLOBOCAN 2018, breast cancer ranks the highest in number of incidences per year and second in number of casualties in Indonesia.[2] To date cancer treatment in Indonesia can cost tens to hundreds of millions rupiah, the high price of treatment not only burdens the patient but also causes significant financial burden towards the country through national healthcare programs and loss of productivity.[3] This fact indicates a new form of treatment that is effective, affordable and widely available as a curative method towards breast cancer is needed.

Surgery is the main treatment modality for the removal of tumor, while radiation therapy is used in cases where surgery could not remove the tumor. Surgery is followed up by adjuvant therapy in order to ensure recovery and decrease risk of metastasis. Adjuvant therapy is in the form of radiation therapy, chemotherapy, hormonal therapy and targeted therapy.[4] However, current treatment still produces adverse physical effects and is also very taxing on the patient's mental. In addition, the cost of treatment is still very expensive and causes a huge financial burden to the patient.[4] Therefore, there is a need to find a form of treatment which is more affordable with less adverse side effect. The solution to this problem could be found by exploring the possibilities of using herb plant that is abundantly found in the wild and easily farmed[5], such as *Clitoria ternatea*.

Clitoria ternatea herb is commonly known in Indonesia as *bunga telang*, it is part of the Fabaceae family of plants. The deep-blue colored flower of the *Clitoria ternatea* has been used for centuries in multiple Asians countries as herbal medicine to cure various conditions.[6] Its anticancer effect is currently explored in the scientific community. The phytochemicals contained in *Clitoria ternatea* displays various effects towards different cells. Pharmacologically, the importance of *Clitoria ternatea* is widely recognized as various research shows that *Clitoria ternatea* displays various effects such as anti-inflammatory, antioxidant and anticancer.[7] Through gas chromatography-mass spectroscopy analysis, multiple phytochemicals such as; N-Hexadecanoic acid, 13-Octadecenal, Oleic acid, Lanosterol, L-Pentadecene, 2-Methyl-z,z-3,13-octtadecadienol, Trimethyl[4-(2-methyl-4-oxo-2-phenoxy)]silane and 2,6-Lutidine 3,5-dicholoro-4-dodecylthio, have been identified to have cytotoxic activity towards cancer cells.[8]

Antioxidant properties and cytotoxicity of *Clitoria ternatea* against several cancer cells have been reported by previous researchers, however, investigation on phytochemical component, antioxidant activity and anti-breast cancer activity of *Clitoria ternatea* collected form Depok, West Java, Indonesia, are still limited. Therefore, in this research we explore further phytochemical component containing in *Clitoria ternatea* originated from Depok, West Java, Indonesia, as well as to explore its antioxidant activity and its anti-breast cancer effect on T47D cancer cells.

2. Results

2.1 Phytocemical Analysis

Clitoria ternatea extracts are labelled according to its solvent which are n-hexane extract (CHE), ethanol extract (CEE), and ethyl acetate extract (CAE). The phytochemicals that were tested were alkaloids, flavonoids, glycosides, saponins, steroids and tannins.

Results of phytochemical analysis of *Clitoria ternatea* extracts is summarized in Table 1. As shown in Table 1, that all three extract of *Clitoria ternatea* contains triterpenoid. CHE results do not show any other phytochemicals except triterpenoid. CEE consists of flavonoids and tannins, while CAE consists of glycosides and tannins

Table 1. Phytocemical screening result of *Clitoria ternatae* extracts

	Glycosides	**Alkaloids**	**Flavonoids**	**Tannins**	**Saponins**	**Triterpenoids/ Steroids**
CHE	-	-	-	-	-	**Triterpenoids**
CEE	-	-	+	+	-	**Triterpenoids**
CAE	+	-	-	+	-	**Triterpenoids**

Thin layer chromatography (TLC) analysis of *Clitoria ternatea* extracts are conducted by placing of the extracts on a straight line drawn 0.5 cm from the bottom edge of a TLC plate. The plate is then put into a chamber containing a mixture (solvent) of n-hexane and ethyl acetate with a ratio of 2:1. The solvent will gradually move up the TLC plate through diffusion. Once the solvent rises into the upper edge of the plate, the TLC plate is put under UV light at 366 nm to observe chemical compound spots formed by the extract as the solvent moves up the plate (Figure 1). The result of the TLC is presented as Rf (retention factor). Rf value is measured by dividing the travel distance of each spot from the initial line by the total distance travelled of the solvent. The Rf value and total phytochemical components is presented in the Table 2. Ethanol extract (CEE) and ethyl acetate extract (CAE) shares the most (4 phytochemical components) with Rf value of 0.07; 0.39; 0.41and 0.95 for CEE, and 0.7; 0.86; 0.93 and 0.98 for CAE, respectively. Whereas n-hexane extract (CHE) has the least (2 phytochemical components) with Rf value of 0.93 and 0.98.

Table 2. Rf value and phytochemical components of *Clitoria ternatea extracts*

	CHE	**CEE**	**CAE**
Rf Value	0.93 and 0.98	0.07, 0.39, 0.41 and 0.95	0.7, 0.86, 0.93, and 0.98
Phytochemical Components	2	4	4

Figure 1. TLC result of *Clitoria ternatea* extracts

2.2 Antioxidant Activity of C. ternatea on DPPH

The result of the antioxidant activity using DPPH assay is presented as the IC50 value of each extract of *Clitoria ternatea.* CHE (n-hexane extract) was not done because of it being a non-polar extract which not soluble in DPPH's polar solvent of ethanol. The test was conducted three times. The IC50 value for ethanol extract (CEE), ethyl acetate extract (CAE) and positive control of ascorbic acid on DPPH are 5.21, 14.15 and 0.69 μg/mL respectively (Table 3).

Table 3. IC50 value of *Clitoria ternatea* extracts and ascorbic acid on DPPH

Extract	IC50 (µg/mL)
CEE	5.209986
CAE	14.15433
Ascorbic acid	0.69

A linear graph was made by using the log concentration of the extract as the X-axis and the inhibition percentage as the Y-axis. By doing so, we can see a linear correlation between the concentration of extract and percent inhibition against DPPH free radical. The results are plotted to obtain the linear regression equation. The linear regression equation for CEE is y = 13.441x + 40.365 with a R^2 value of 0.9557 (Figure 2) and the linear regression equation for CAE y = 20.24x + 26.706 with a R^2 value 0.9509 (Figure 3). To obtain the IC50 value we substitute the Y value of the linear regression equation by 50 and inserting them into 10^ ((50-40.365) / (13.441) for IC50 of CEE and 10^ ((50-26.706) / (20.24)) for IC50 of CAE. Hence, the IC50 value of CEE and CAE are 5.209986 µg/mL and 14.15433 µg/mL respectively.

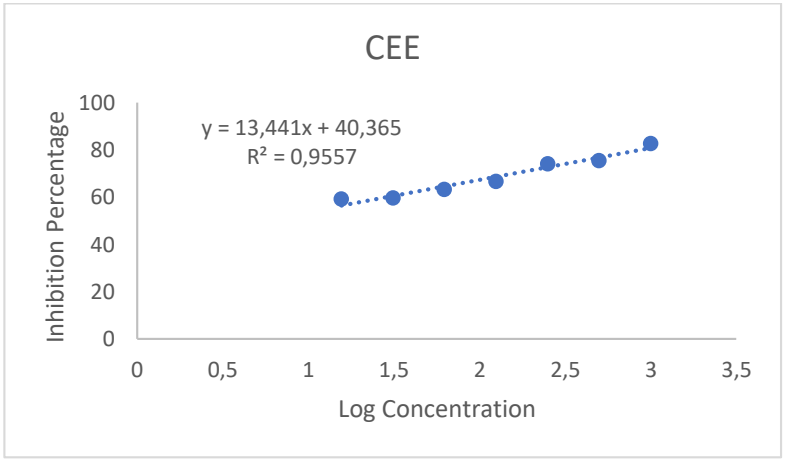

Figure 2. Linear graph of ethanol extract of *Clitoria ternatea* on DPPH

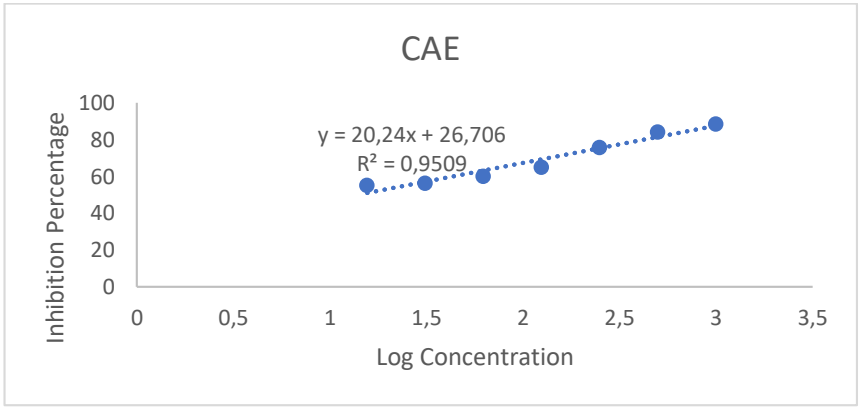

Figure 3. Linear graph of ethyl acetate extract of *Clitoria ternatea* on DPPH

2.3. Cytotoxicity of C. Ternatea Extract

The results of MTT assay for *Clitoria ternatea* extracts and doxorubicin (positive control) towards T47D breast cancer cells is shown on Table 4. The test was conducted twice and the average IC50 values for CEE, CAE and CHE are 1.27839452, 22.5758815 and 32.3829074 respectively. Doxorubicin gave an IC50 value of 1.00 µg/mL.

Table 4. IC50 (μg/mL) of *Clitoria ternatea* extract and doxorubicin on T47D cells

	IC50 value (μg/mL)			
	CEE	**CAE**	**CHE**	**Doxorubicin**
Trial 1	1.4807388	10.3632739	10.4576505	1.00
Trial 2	1.07605024	34.788489	54.3081643	1.00
Mean	1.27839452	22.5758815	32.3829074	1.00

The IC50 of *Clitoria ternatea* extracts on breast T47D cells by MTT assay is achieved by doing a linear regression the same with DPPH assay. The equations for each ethanol extract tests are as follows y = 50.851x - 41.331 (R^2 = 0,9906) and y = 8.7332x + 49.722 (R^2 = 0,9923) consecutively (Figure 10).

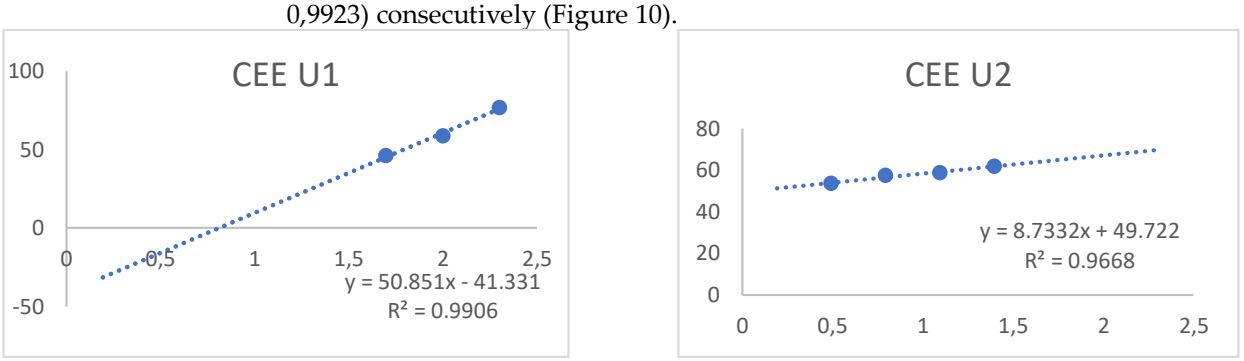

Figure 4. Linear graph of ethanol extract of *Clitoria ternatea* on T47D cells

3. Discussion

3.1 Phytochemical constituents of C. ternatea

The test performed to find out the secondary metabolites of *Clitoria ternatea* is thin layer chromatography and phytochemical analysis. The phytochemicals being tested for are saponin, flavonoid, tannin, glycoside, triterpenoid, steroid, and alkaloid. These phytochemicals are commonly found in plants and has been proven to be the reason why herbal medicine possess beneficial medicinal properties.

The screening result shows that *Clitoria ternatea* extract contains glycosides, flavonoids, tannins and triterpenoids. More specifically, n-hexane extract (CHE) shows the presence of triterpenoid only, ethanol extract (CEE) contains flavonoids, tannins and triterpenoid while ethylacetate extract (CAE) shows the presence of glycosides, tannins and triterpenoids. This result is further supported by a study done by Mohan et al (2013) which conducted a more in-depth analysis of the phytochemical constituents of *Clitoria ternatea* which shows the presence of glycosides, flavonoids, tannins and triterpenoids in addition to alkaloids, steroids and phenol.[9]

Glycosides is a term coined for any variety of naturally occurring substance which contains a carbohydrate portion, consisting of one or more sugar, combined with hydroxy compound or another carbohydrate. A review done by Khan et al (2019) shows that multiple studies has concluded that various plant derived glycoside isolates demonstrates patent cytotoxic effects against various cancer cell lines in initial preclinical studies.[10]

Flavonoids are phytochemicals found in fruits, vegetables and many other plants which consists of a variable of phenolic structures. Depending on the phenolic groups and structures flavonoids can be further classified into smaller classifications. Various studies have shown that flavonoids display anti-inflammatory activities, through the inhibition of COX-2, and antioxidant properties through direct scavenging of free radicals.[11] Ren et al (2003) has also concluded that the anticancer mechanism of flavonoids is through the inhibition of certain P450 isozymes which are responsible for the production of a number of procarcinogens.[12]

Tannins are plant polyphenols comprised of two or three phenolic hydroxyl groups on a phenyl ring with a moderately large molecule size. Multiple studies have found multiple anticancer properties of tannins. Tannins can inhibit the mutagenicity of Trp-P-1,

MNNG and N-OH-Trp-P-2 which are known as direct-acting mutagens. Tannins, ellagitannins and their oxidized congeners, also shows significant tumor promotion inhibition properties.[13]

Triterpenoids is a part of a larger classification called terpenoids or terpenes. Out of all the terpenes, triterpenoids have been recognized as a unique group of phytochemicals with multiple health benefits, one of them being anticancer. A study by Bishayee et al showed that multiple natural and semi-synthetic triterpenoids have tumor inhibitory properties and even cytotoxicity towards breast cancer cells, including T47D.[14]

The phytochemicals that were found in the preliminary phytochemical screening of *Clitoria ternatea* can be attributed to the antioxidant and cytotoxic property of *Clitoria ternatea*. This is further supported by multiple studies conducted on the phytochemicals as mentioned on the paragraphs above.

The TLC result showed that there are 2 phytochemical components found in CHE, 4 in CEE and 4 in CAE, with a total of 10 phytochemical components found. The Rf values of each extract can be seen in Table 2. CHE and CAE share a phytochemical component with a Rf value of 0.93. These results prove that there are still more phytochemicals to be found and more extensive research can be done to explore it.

3.2 Antioxidant activity of Clitoria ternatea extracts

The phytochemicals found on *Clitoria ternate* will influence the result of DPPH test. The antioxidant activity will be measured by the radical scavenging ability of the solution using DPPH assay. The result will be interpreted as IC50, the concentration amount of the extract to have a 50% inhibition.

The result of the IC50 is compared to the ascorbic acid, the positive control. CHE was not done in consideration because it is a non-polar extract which could not dissolved well in DPPH's polar solvent of ethanol. CEE was presented with an IC50 result of 5.209986 μg/mL while CAE has an IC50 value of 14.15433 μg/mL against DPPH free radical. This means that CEE is a better antioxidant in comparison to CAE as a lower concentration of it can inhibit the extract by 50%. Despite CAE being a weaker antioxidant in comparison to CEE, according to classification of antioxidant activity based on IC50 value by Marjoni et al (2017), both CEE and CAE falls under the category of very active antioxidant as they both have an IC50 value of <50 μg/mL.[15]

3.3 Cytotoxic activity of C. ternatea extracts

Similar to antioxidant activity of *Clitoria ternatea* on DPPH, the cytotoxicity of of *Clitoria ternatea* on T47D cells by MTT assay will be influenced by the phytochemicals contained in the extract. The cytotoxicity of the *Clitoria ternatea* extract will be measured by its IC50 value. Ethanol extract (CEE), ethyl acetate extract (CAE) and n-hexane extract (CHE) displayed cytotoxic activity at a varying level against breast T47D cells. CEE with an IC50 value of 1.27839452 μg/mL is considered to have cytotoxic activity based on American National Cancer Institute, as it has an IC50 value of less than 20 μg/mL.[16] This is further enforced by Atjanasuppat et al (2009) classification which classifies CEE to be very active.[17] Using Atjansuppat et al classification, CAE (IC50 value of 22.5758815 μg/mL) and CHE (IC50 value of 32.3829074 μg/mL) can be classified as active as it has an IC50 value in the range of 20-100 μg/mL.

4. Materials and Methods

4.1 Materials

The sample used for this research is 200 grams of dried *Clitoria ternatea* collected from Depok, West Java, Indonesia. The dried sample was macerated and dissolved in three different solvents which are polar solvents (ethanol), semi-polar solvent (ethyl acetate) and non-polar solvent (n-hexane) in order to get the corresponding extracts of *Clitoria ternatea*. The extracts are then to be tested for antioxidant activity using 2,2-diphenyl 1-picrylhydrazyl (DPPH) reagent and evaluated for anticancer effect against T47D breast cancer cell line using 3-(4,5-dimethylthiazol-2yl)-2-5-diphenyltetrazolium bromide (MTT) assay.

The chemicals and tools used for phtochemical sreening as follows: hot water, tube, HCl 2N, concentrated HCl, Mg strip, $FeCl_3$ 10% solution, sodium acetate hydrate, H_2SO_4, $CHCl_3$, glacial acetic acid, NH_4OH, Dragendroff reagent, Meyer reagent, Thin light chromatography (TLC) plates, TLC developing chamber, UV lamp and microcaps.

The chemicals and tools used for DPPH and MTT assay are stated as follows: MTT reagent, DPPH reagent, DMSO, Microplate 96 well, DPBS, UV-Visible Spectrophotometer, ascorbic acid, ethanol, T47D cell line, doxorubicin.

4.2. Research Design

This research is an *in vitro* experimental research with Breast T47D cancer cells. It consists of several steps in order to find out antioxidant activity, cytotoxicity and phytochemistry of *Clitoria ternatea* towards breast T47D cancer cells. The first step is to collect and dry the *Clitoria ternatea* sample. Once dry, the sample is macerated in preparation for sample extraction. The extract is then used for phytochemistry testing and thin layer chromatography (TLC) analysis, in order to find out the phytochemical metabolites contained in *Clitoria ternatea*. The next step is antioxidant testing of *Clitoria ternatea* extract which is done through DPPH method. The last step is in vitro cytotoxicity evaluation of the extracts against breast T47D cancer cells using MTT assay.

4.3 Phytochemical Analysis of Clitoria ternatea extract

4.3.1 Saponin Screening

Place 1 gram of extract in a test tube and dilute it with 10ml aquadest. After inserting the extract, shake the test tube vertically to mix for 10 second and wait for another 10 seconds. Observe if there are any froth that is 1-10cm in height and is stable for 10 minutes, this indicates that saponin might be present in the extract. Add 1 drop of 2N chloric acid into the frothy mixture. If the froth is still present after the addition of chloric acid it is confirmed that saponin is present.

4.3.2 Flavonoid Screening

Insert 1ml of *Clitoria ternatea* extract into a test tube. Add 0.5ml of concentrated chloric acid and 4cm of magnesium strip into the test tube. Observe the reaction, if the color red, orange or green is formed, flavonoid is present.

4.3.3 Tannin Screening

Insert 1ml of *Clitoria ternatea* extract into a test tube. Add 1ml of $FeCl_3$ 10% into the test tube. Observe change, if black/dark green/dark blue precipitate forms result is positive.

4.3.4 Glycosides Screening

Insert 0.1ml of *Clitoria ternatea* extract into a test tube. Place test tube on top of a water bath to evaporate. Add 1 ml of acetic anhydrate and 2ml of concentrated sulfuric. Wait and observe change, if blue or green color forms result is positive.

4.3.5. Steroid and Triterpenoid Screening

Insert 2 ml of *Clitoria ternatea* sample in a porcelain glass to be evaporated and move evaporated sample into a test tube. Add 0.5 ml of chloroform and acetic anhydrate into the evaporated sample. Slowly drip down 2 ml of sulfuric acid into the test tube. Observe change, if the sample contains triterpenoid, a violet or brown ring would form on top of the mixture. If Steroid is present, a blue or green ring would form instead.

4.3.6 Alkaloid Screening

Insert 2 ml of *Clitoria ternatea* sample into a porcelain glass to be evaporated. Add 5 ml of 2N hydrochloric acid and divide sample into 3 different test tubes. The first tube is blank, add 3 drops of Dragendorff reagent into the second tube and add 3 drops of Mayer reagent into the third tube. A positive alkaloid test is indicated if an orange precipitate is formed in the second test tube and a yellow precipitate is formed in the third test tube.

4.4 Qualitative Analysis Using Thin Layer Chromatography (TLC)

Prepare samples derived from all 3 solvents (n-hexane, ethanol and ethyl acetate). Prepare the TLC strip after preparing the samples. Measure 1 cm from the end of the strip and place a drop of sample as starting point. Place the TLC strip into eluent container without touching the starting point. Wait until the mobile phase has ended. Remove and dry the TLC strip. Observe using ultraviolet light (366nm). Measure the retardation factor.

4.5 Antioxidant Activity Test Using DPPH Method

Prepare *Clitoria ternatea* extract that has been dissolved in three solvents (n-hexane, ethanol and ethyl acetate) with concentration of 3.75, 6.25, 12.5, 25 and 50 μg/ml. Take 100 μl of each solvents and insert them into a test tube. Add 2.9 ml DPPH with a concentration 0.004% in a methanol solvent into a test tube. Repeat into a blank test tube and another test tube containing water and vitamin C as the positive control. Incubate the test tube in a dark environment and room temperature for 30 minutes. Measure the absorbance using photospectrometry with a wavelength of 515nm.

4.6 Cytotoxicity Test Against Breast T47D Cancer Cells Using MTT Assay

Culture and harvest T47D breast cancer cells according to procedure. Count the number of cells and dilute using culture medium in accordance to cell counting procedure. Transport 100 μl of culture medium and cells into the well plates, leave 3 well plates empty to act as control. Observe cell distribution using microscope. Incubate the cell culture for 24 hours. Prepare 8 different concentrations of *Clitoria ternatea* samples that have been dissolved in n-hexane, ethanol, methyl acetate into 51.2, 25.6, 12.8, 6.4, 3.2, 1.6, 0.8 and 0.4 μg/ml respectively. Discard culture medium from the well plates by flipping it over to decant its content. Add 100 μl of phosphate-buffered saline into the well plates containing cells. Decant the phophate-buffered saline. Each 3 extract is then inserted into the well plates 3 times. Place well plate into a carbondioxide incubator for 24 hours to measure sample cytotoxicity. Add MTT reagent with a concentration of 5 mg/ml into the testing well plates and a 100 μl into the control. Incubate the well plates for 4 hours. Observe the cells using a microscope to find purple color from formazan. Add 100 μl stopper, sodium dodecy sulfate with 10% concentration dissolved in HCl 0.1 N, into all the well plates. Wrap the well plates with aluminium and incubate for 24 hours in a dark environment. Insert the well plates into ELISA reader with a wavelength of 630 nm to find the absorbancy of the solvents in the well. Count the percentage of cells that are still alive and IC_{50} value.

5. Conclusions

Clitoria ternatea containing 4 phytochemicals constituents that are identified with preliminary phytochemical screening: glycosides, flavonoids, tannins and triterpenoids. Whereas TLC analysis was able to identify 10 phytochemical constituents of *Clitoria ternatea*. Both ethanol extract (CEE) and ethyl acetate extract (CAE) of *Clitoria ternatea* demonstrate a strong antioxidant activity against DPPH. MTT assay of *Clitoria ternatea* shows that ethanol extract (CEE) resulted in a very active cytotoxic activity towards T47D breast cancer line.

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